FORM PTO 1390 (REV 10-2000) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER HO-P02222US0 (10105125)
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 09/869179 To be assigned
INTERNATIONAL APPLICATION NO. PCT/GB99/04352	INTERNATIONAL FILING DATES 22 December 1999	PRIORITY DATE CLAIMED 22 December 1998
TITLE OF INVENTION	METHOD OF SEQUENCE IDENTIFICATION	
APPLICANT(S) FOR DO/EO/US	Jeffrey Errington	

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This is an express request to promptly begin national examination procedures (35 U.S.C. 371 (f)).
4. [X] The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371 (c)(2))
   a. [X] is attached hereto (required only if not communicated by the International Bureau).
   b. [ ] has been communicated by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] An English language translation of the International Application as filed (35 U.S.C. 371 (c)(2)).
7. [x] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. [x] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. [ ] An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11 to 16 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 & 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information:
    Return postcard

U.S APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/869179 To be assigned	PCT/GB99/04352	HO-P02222US0 (10105125)

17. [X] The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) – (5)):

		CALCULATIONS PTO USE ONLY
[ ]	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1000.00	
[X]	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $860.00	
[ ]	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $710.00	
[ ]	International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) $690.00	
[ ]	International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00	

ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 860.00	
Surcharge of $ 130.00 for furnishing the oath or declaration later than [ ] 20 [X] 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	$ 130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	18-20 =		x	$ 0.00	
Independent claims	3-3 =		x	$ 0.00	
MULTIPLE DEPENDENT CLAIM(s) (if applicable)			x	$	

TOTAL OF ABOVE CALCULATIONS =	$ 990.00	
[X] Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are rduced by ½.	$ 495.00	
SUBTOTAL =	$ 495.00	
Processing fee of $ _____ for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +	$	
TOTAL NATIONAL FEE =	$ 495.00	
Fee for recording the enclosed assignment (37 CFR 1.21 (h)). Assignment must be accompanied by appropriate cover sheet (37 CFR 3.28, 3.31) + (per property).	$	
TOTAL FEES ENCLOSED =	$ 495.00	
Amount to be Refunded:	$	
Charged:	**$495.00**	

a. [ ] A check in the amount of $ _____ to cover the above fees is enclosed.

b. [X] Please charge my Deposit Account No. 06-2375 (order 10105125) in the amount of $ 495.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required or credit any overpayment to my Deposit Account No. 06-2375. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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SIGNATURE:

Melissa L. Sistrunk
NAME

45,579
REGISTRATION NUMBER

DATED: June 21, 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Title: Method of Sequence Identification		§	**Docket No.: P02222US0 (10105125)**
		§	
		§	**Examiner: Not yet assigned**
Inventors:	**Jeffrey Errington**	§	
		§	**Group Art Unit: Not yet assigned**
Filed:	**June 21, 2001**	§	
		§	**Customer No. 26271**

Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Dear Sir:

Applicants submit a preliminary amendment to a U.S. Patent Application filed herewith under 35 U.S.C. §371. Please make the following amendments.

In the specification:

Please add the following paragraph immediately following the title on Page 1:

- -This application claims priority to PCT/GB99/04352, filed December 22, 1999, which claims priority to European Patent Application No. 98310567.7 filed December 2, 1998.- -

In the specification:

Please add the following claims:

19. A method for identifying a regulatory sequence which is affected by a feedback mechanism on alteration of synthesis or activity of a bacterial essential protein, comprising:

i) providing a bacterial cell having a reporter gene under the control of a candidate regulatory sequence;

ii) selecting a target essential protein which is expressed in the cell;

iii) altering the synthesis or activity of the essential protein;

iv) monitoring expression of the reporter gene; and

v) determining thereby whether the candidate regulatory sequence is affected by a feedback mechanism responsive to alteration of the synthesis or activity of the essential protein.

20. A method of identifying a regulatory sequence whose activity is affected by a feedback mechanism or an alteration of the synthesis or activity of an essential bacterial protein, comprising:

(a) monitoring expression of a bacterial gene in a bacterial host cell in the presence of normal and altered synthesis or activity of the essential protein;

(b) identifying differential gene expression in the presence of normal and altered synthesis or activity of the essential protein; and

(c) identifying thereby a regulatory sequence whose activity is affected by the feedback mechanism.

21. The method of claim 20, wherein step (b) comprises:

(a) providing an array of nucleotide sequences of the nucleic acid sequences of the bacterial cell;

(b) recovering polynucleotide material from the cells;

(c) applying said polynucleotide material to the array; and

(d) monitoring for hybridization of the bacterial nucleic acid material to the array.

22. The method according to claim 20, wherein step (b) comprises recovering and separating proteins from the bacterial cell and monitoring for a change in concentration of a protein in the presence of normal and altered synthesis or activity of the essential protein.

23. A method for identifying a modulator of a bacterial essential protein, comprising:

i) providing a bacterial host cell which expresses the essential protein, wherein the cell comprises a polynucleotide construct comprising a regulatory sequence operably linked to a reporter nucleic acid sequence, wherein the regulatory sequence is associated with a feedback mechanism responsive to alteration in the synthesis or activity of the essential protein and is identified according to the method of claim 19;

ii) contacting a test substance with the host cell; and

iii) monitoring expression of the reporter gene to determine thereby whether said substance modulates the synthesis or activity of the essential protein.

24. The method of claim 23, wherein the essential protein is involved in cell wall synthesis, teichoic acid synthesis, DNA replication, RNA synthesis, cell division, chromosome segregation, translation or lipid synthesis.

25. The method of claim 23, wherein inhibition of the essential protein up-regulates expression of the reporter nucleic acid sequence from the regulatory sequence.

26. The method of claim 23, wherein the regulatory sequence has the activity of a promoter for the nucleic acid sequence encoding the essential protein and inhibition of the essential protein up-regulates expression from its promoter.

27. The method of claim 23, wherein the regulatory sequence has the activity of a promoter for a gene which does not encode the essential protein but which is up-regulated via the feedback mechanism in response to alterations to the synthesis or activity of the essential protein.

28. The method of claim 23, wherein the reporter gene comprises a nucleic acid sequence which is up-regulated in response to alterations in the synthesis or activity of the essential protein, and wherein step (iii) comprises monitoring for differential expression of the gene in the presence or absence of the test substance.

29. The method according to claim 23, wherein the bacterial cell is provided with a second polynucleotide construct comprising a promoter operably linked to a second reporter gene and the method further comprises monitoring expression of the second reporter gene.

30. The method of claim 23, further comprising determining whether the test substance demonstrates specific inhibition of the essential protein.

31. The modulator of claim 23, wherein said modulator is an inhibitor of the bacterial essential protein.

32. The inhibitor of claim 31, wherein said inhibitor is for use in a treatment of an animal.

33. The inhibitor of claim 32, wherein said animal is a human.

34. The inhibitor of claim 32, wherein said inhibitor is an antibiotic.

35. A pharmaceutical composition comprising the inhibitor of claim 32 and a pharmaceutically acceptable carrier.

36. A method of inhibiting bacterial growth comprising identifying an inhibitor of claim 31, and contacting bacteria with said inhibitor.

Please cancel claims 1-18.

Applicants address fees in the Transmittal Letter to the United States Designated Elected Office Concerning a Filing under 35 U.S.C. § 371 submitted herewith. If at anytime during the prosecution of this application additional fees are required, the Commissioner is authorized to withdraw the fees from the deposit account of Fulbright & Jaworski L.L.P., No. 06-2375 under Order No. 10105125.

Respectfully submitted,

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METHOD OF SEQUENCE IDENTIFICATION

Introduction

There is an urgent need to identify and develop new families of antibiotics to combat the continuing threat of antibiotic resistance. Most major pharmaceutical companies are using the following general approach to identify new antibiotics.

1. Target identification. Genes that appear to be essential for bacterial viability are first identified. Most often, this is based on inviability of the cells containing a form of the gene that has been inactivated. Information from genomic DNA sequence is used to identify genes that are conserved in bacteria but not in humans. The targeting of such genes is thought to be more likely to provide inhibitors that will be specific to bacterial cells - a crucial feature for efficacy as an antibiotic.

2. Assay development. A suitable means of screening for inhibition of the function of the target gene is then devised. This can be either an *in vitro*, biochemical assay, or a whole cell assay.

3. High throughput screening. The assay is configured in such a way that a very large number of chemical compounds can be screened for activity against the target function. Many pharmaceutical companies have access to very large collections of compounds that can be screened. Compounds detected in this way provide lead molecules from which antibodies can potentially be derived.

Most pharmaceutical companies now have good resources with which to underpin activities 1 and 3. However, there is a major bottleneck in the development of assays, especially for the large number of genes of unknown function that are potentially good targets.

Most important functions in living cells, especially bacteria and lower eukaryotes, are tightly regulated. Feedback mechanisms often ensure that products are made only in amounts sufficient to fulfil their functions. There are numerous examples of feedback regulation at the level of transcription or translation; for example, transcription attenuation to control tRNA synthetase (Henkin, 1994) and ribonucleotide precursor synthesis (Lu *et al*, 1996); DNA supercoiling controls the promoter for DNA gyrase (Menzel & Gellert, 1983). The same is likely to occur at

the promoters of many other genes, including genes for cell division, DNA replication, etc.

The present invention makes use of this feedback regulation, hereinafter sometimes called autoregulation, to provide whole cell assays for screening compounds for antibiotic or other biological activity. It may be convenient for this purpose to make use (to effect expression of a reporter gene) of a promoter for the target gene. Alternatively other regulatory sequences, e.g. promoters of other genes of known or unknown function, may be used for the purpose. It is another object of this invention to provide a means of identifying such regulatory sequences.

The Invention

In a first aspect, the invention provides a method for identifying a modulator of a bacterial essential protein comprising:

i) providing a bacterial host cell which expresses the essential protein and having a polynucleotide construct comprising a regulatory sequence operably linked to a reporter gene wherein the regulatory sequence is associated with a feed back mechanism responsive to changes in the synthesis or activity of the essential protein;

ii) contacting a test substance with the host cell; and

iii) monitoring expression of the reporter gene to determine thereby whether the said substance modulates the synthesis or activity of the essential protein.

In another aspect, the invention provides an inhibitor of a bacterial essential protein identifiable by the method of the invention; an inhibitor of a bacterial essential protein identified according to the method of the invention; an inhibitor as defined above for use in a method of treatment of a human or animal body; or an inhibitor as defined above for use as an antibiotic.

The invention also provides a pharmaceutical composition comprising the inhibitor as defined above and a pharmaceutically acceptable carrier.

In another aspect, the invention provides a method for identifying a

regulatory sequence which is affected by a feedback mechanism on alteration of synthesis or activity of a bacterial essential protein comprising:

i) providing a bacterial cell having a reporter gene under the control of a candidate regulatory sequence;

ii) selecting a target essential protein which is expressed in the organism;

iii) altering the synthesis or activity of the essential protein; and

iv) monitoring expression of the reporter gene.

v) determining thereby whether the candidate regulatory sequence is affected by a feedback mechanism.

The invention also provides a method of identifying a regulatory sequence whose activity is affected by a feedback mechanism on alteration of the synthesis or activity of an essential bacterial protein comprising monitoring gene expression in the presence of normal and altered synthesis or activity of the essential protein, and identifying thereby a regulatory sequence whose activity is affected by a feedback mechanism associated with the essential bacterial protein.

In another aspect the invention provides cells of an organism suitable for screening compounds for biological activity, which cells contain a chromosome including:

a) a target gene whose expression or activity is subject to a feedback mechanism, and

b) an artificially introduced reporter gene under the control of a regulatory sequence associated with the said feedback mechanism, whereby a reduction of synthesis or activity of a target gene expression product is associated with an increase in the expression of the reporter gene.

In another aspect the invention provides a method of screening compounds for biological activity, which method comprises incubating the compounds with aliquots of the cells as defined, and observing the level of expression of the reporter gene.

In another aspect, the invention provides compounds e.g. antibiotics identified by the method; and use of the compounds so identified to treat, e.g. kill or inhibit the growth of, bacteria.

The invention provides a method for screening for an inhibitor or modulator of a bacterial essential protein. The essential protein is encoded by a target gene and may be of known or unknown function. Alteration of expression or activity of the target essential protein leads to a feedback mechanism in the bacteria, which serves to alter or up regulate expression from a promoter or regulatory sequence of a bacterial gene.

The nature of the feedback mechanism is not critical; it may operate at the RNA level or at the protein level and may involve transcription and/or translation of the target gene and/or activity of a target gene expression product. The target gene will generally encode a protein that is essential for viability and well conserved in bacteria or other micro-organisms but absent from or highly dissimilar in higher organisms e.g. mammals or humans.

When expression of the target gene or synthesis or activity of a target gene expression product is altered (the means by which that alteration is effected being unimportant for present purposes), then a feedback mechanism may operate in the bacterial cell to compensate for the alteration. The feedback mechanism may operate on a regulatory sequence e.g. a promoter of the target gene. Thus, for example a reduction of synthesis or activity of a target gene expression product may be associated, via the feedback mechanism, with an increase in activity of the promoter of the target gene. In accordance with an assay of the invention, a polynucleotide construct is provided in the bacterial cell comprising a reporter gene under the control of the promoter of the target gene.

Alternatively or additionally, a reduction in the synthesis or activity of the essential protein may be associated with an alteration, and generally an increase in the expression of another bacterial gene, for example, through up regulation of the promoter for that gene. In an alternative embodiment of the invention, a polynucleotide construct is provided comprising a reporter gene under the control of the promoter or other regulatory sequence that is not associated with the target essential protein *per se* but is subject to upregulation through a feed back mechanism when the activity or synthesis of the essential protein is altered.

In some instances, it may not be possible to predict which genes will be

involved in a feed back mechanism other than a promoter of the target gene. The invention also provides methods of identifying potential genes which are subject to a feed back mechanism effected by alterations in the activity or synthesis of the essential functional protein under investigation. Once such a gene has been identified, a reporter gene under the control of the promoter or regulatory sequence for that gene may be readily constructed and incorporated in a bacterial cell for use in accordance with an assay of the invention.

The cells of the invention thus contain an artificially introduced reporter gene under the control of a regulatory sequence associated with the target gene that is to say affected by a feedback mechanism associated with a reduction in the synthesis or activity of the target gene product. In an alternative aspect of the invention the reporter gene comprises the gene whose expression is altered by the feedback mechanism described above and the assay comprises monitoring for up regulation for that gene, for example using a gene array or proteomics technique to monitor transcription or expression of the gene.

The target gene in accordance with the present invention encodes a protein which is essential for bacterial viability such that a test substance which effects the essential protein, either effecting its synthesis or activity may be useful as an antibiotic. As highlighted above, in accordance with the present invention, such a target gene must also in some way be associated with a feedback mechanism effecting either the expression of the target gene itself or other genes within the bacterial cell. The promoters or other regulatory sequences of the target gene or genes affected by the feedback mechanism may thus be useful when coupled to a reporter gene in an assay. By monitoring for expression of the reporter gene, a substance which inhibit synthesis or activity of the target protein may be identified. Proteins involved in cell wall precursor synthesis, teichoic acid synthesis, DNA replication, RNA synthesis, cell division, chromosome segregation, translation and other essential genes are all suitable targets for use in accordance with the invention. Some specific examples are discussed in more detail below, by way of example only.

The following are envisaged as preferred target genes for use in *B. subtilis* or other bacteria. These may include proteins involved in the following

process:

Cell wall (peptidoglycan) precursor synthesis (including the products of the genes: *murA,B,C,D,E,F,G,Z, dal, dap, ddlA*);

Teichoic acid synthesis (*tagA,B,C,D,E,F,G,H, gtaB*);

DNA replication (*dnaA, gyrA,B, topA,B, lig[yerGJ*);

RNA synthesis (*sigA, rpoA, rpoB, rpoC*);

Cell division (*fisA,L,W,Z, divIB, divIC, divIVA, pbpB);*

Chromosome segregation (*spoIIIE, soj, spoOJ codV, ripX*);

Translation (*infA, B, C, fmt, efp*):

Miscellaneous (*obg, lgt);*

Essential genes of unknown function discovered by the inventor (*yjbN, yloQ*).

Lipid synthesis *(pgsA).*

Examples of target genes are discussed in more detail below.

DnaA - an example of a DNA replication protein

The *dnaA* gene is widely conserved among bacterial species and in *E.coli* and *B. subtilis* at least, it is essential, being required for initiation of DNA replication (although the Cyanobacterial gene was recently reported to be non-essential; Richter *et al.*, 1998, *J. Bacteriol.* **180**, 4946-4949). Eukaryotes, in contrast, use a quite different mechanism to initiate chromosome replication, so agents that specifically inhibit DnaA function should have selective toxicity against bacteria. Accumulation of DnaA during the cell cycle is thought to result in gradual filling of binding sites ("DnaA boxes") in the *oriC* region. Eventually, enough DnaA accumulates to allow formation of an initiation complex that leads to the initiation of bidirectional chromosome replication. In *B. subtilis* at least, the *dnaA* gene is monocistronic and located right next to *oriC* (Moriya *et al.*, 1988, EMBO J. 7, 2911-2917). There is a long upstream regulatory region containing several DnaA boxes. Two of these appear to overlap the promoter region of the gene. There is well-documented evidence in both *E.coli* and *B. subtilis* that DnaA negatively autoregulates, so depletion of DnaA is compensated for by increased transcription from its promoter. Preliminary experiments are in progress to show that depletion of DnaA protein (by

artificially repressing its gene) result in increased transcription from the *dnaA* promoter. If this works, reporter genes coupled to the *dnaA* promoter should provide a convenient means of screening for inhibitors of this excellent antibiotic target. Promoters to genes uninvolved with DNA replication (e.g. the P_{xyl} promoter, induced by the presence of xylose) should be either unaffected by inhibitors of DnaA, or else gradually shut down, as the cells begin to die through loss of their DNA. It is possible that the arrest in initiation of DNA replication caused by depletion or inhibition of DnaA will result in repression of genes involved in other steps in DNA replication. The promoters of such genes might provide better control promoters to ensure the specificity of inhibitors against DnaA, rather against DNA replication in general. A dual reporter system based on the P_{dnaA} and possibly P_{xyl} promoters should provide a sensitive and specific whole cell assay for inhibitors of DnaA function.

Dal - an example of a protein involved in a cell wall synthesis

Bacterial cell walls are essential stress-bearing structures made of peptidoglycan (PG). A wall is present in virtually all eubacteria. It is composed of long glycan (amino sugar) polymers crosslinked by short peptides. The peptides are unusual in that they contain amino acids in the D-isomeric state. There is no structure equivalent to the cell wall in higher eukaryotes and D-amino acids are generally absent. Many important antibiotics, including penicillins, cephalosporins and vancomycin act on PG synthesis. D-alanine, which is universally found in PG, is obtained by bacteria either from their growth medium or by the action of an enzyme, D-alanine racemase, encoded by the *dal* gene, which converts L-alanine to the D-form. *dal* is a very highly conserved gene in eubacteria but absent from eukaryotes.

Tests are in progress to determine whether the *dal* promoter is autoregulated; its promoter is expected to be up-regulated in response to depletion of the Dal protein. If this is the case, the promoter can be used to screen for inhibitors of Dal. P_{xyl} can again be used as a control, although there might be better promoters (e.g. one responding negatively to increases in the substrate used by Dal).

InfC - an example of a protein involved in translation

The process of translation initiation is highly conserved in bacteria but differs fundamentally from eukaryotes. Thus, initiation codons are defined by a "ribosome-binding site" (RBS), which lies close to the initiation codon, which is usually AUG but sometimes UUG or GUG.

In humans, the first AUG in the mRNA tends to be used, irrespective of the context - there is no equivalent of the RBS. Translation initiation factor 3 of bacteria is highly conserved in bacteria and seems to be required for specificity of initiation at start codons. In both *B. subtilis* and *E.coli*, the initiator codon of the *infC* gene is highly unusual - AUU. Probably, this codon can only be used to initiate translation and thus make more InfC protein, when the factor is limiting, allowing the ribosome to initiate less stringently. Experiments with *E.coli* support this (reviewed by Grunberg-Manago, 1996, In *Escherichia coli* and *Salmonella typhimurium: cellular and molecular biology*, eds Neidhardt *et al.*, ASM Press, Washington D.C. pp 1432-1457). A highly specific whole assay for inhibitors of InfC is envisaged based on a use of a pair of reporter genes. Both will carry a promoter driving transcription of the *infC* translation initiation region: in one case, the reporter gene will be fused in frame with the beginning of the *infC* coding region, including the AUU initiation codon: the other will have a different reporter gene fused to an identical leader except with AUG as initiator. The first reporter should induce when InfC is depleted or inhibited. The latter will provide a control essentially unresponsive to changes in the levels of InfC.

Fmt - an example of a protein involved in the translation

Methionyl-tRNA formyltransferase (Fmt) carries out the final step in synthesis of the initiator tRNA in bacteria. Bacterial cells use a special initiator tRNA, $tRNA_{Fmt}$, specifically at start codons. Eukaryotic cells use the normal tRNA for methionine at both initiator and internal codons and so have no need of Fmt. Disruption of the *fmt* gene of *E.coli* (at least) causes a severe growth defect (Guillon, J.-M., Mechulam, Y., Schmitter, J.-M., Blanquet, S., Fayat, G. (1992) J. Bacteriol, **174**, 4294-4301), so its product is likely to be a good target for antibiotics. In the gene sequence, there is an unusual RBS with an ATG right next to it, followed by an

Ile codon and a stop. The correct initiator AUG follows. In the presence of excess Fmt. binding is expected to occur at the inappropriate upstream AUG and this might block use of the correct start codon. This could be tested with wild type and mutant initiator regions fused in frame to *lacZ*. as for *infC*. It is expected that these reporters will respond appropriately to depletion of Fmt. and will provide a specific assay for inhibitors of Fmt.

In one aspect of the invention a second polynucleotide construct is provided in the bacterial host cell comprising a second reporter gene linked to a second promoter. The second promoter is provided such that under the conditions of the assay. in the absence of a test substance the second reporter gene would normally be expected to be expressed. This allows for non specific effects of the test substance to be identified. For example. if the test substance is a general inhibitor of translation within the bacterial cell. no expression of the second reporter gene would be expected.

The first and/or second reporter genes encode products which can readily be detected. For example, the reporter product may be detected by fluorescent, luminescent or other standard reporting techniques. The reporter gene products may comprise an enzyme such as β-galactosidase. production of which may be identified by use of colorogenic or fluorogenic enzymes substrates. Other reporter genes include β-glucuronidase, green fluorescent protein (GFP) and variants thereof. luciferase. chloramphenicol acetyl transferase. catechol oxidase, an antigen which may readily be recognised by an antibody, other affinity ligands such as streptavadin/biotin or protein A which may be detected by antibodies etc. The first and second reporter genes where present are selected such that it is possible to differentiate between expression of the first reporter gene and expression of the second reporter gene. In an alternative aspect of the invention, no second reporter gene is provided. However. other properties such as the optical density of the bacterial culture may be monitored with a view to assessing to what extent non-specific effects are occurring based on changes in culture growth which may be taken into account when assessing the effect of the test substance.

The polynucleotide construct(s) comprise a promoter operably linked to the reporter gene to be expressed. Preferably, where a second polynucleotide construct is provided, the second promoter comprises an inducible promoter. The conditions required to induce this promoter can be applied to the host cell when adding the test substance during the course of the assay. Such inducing conditions would allow for the expression of the second reporter gene in the absence of the test substance. Examples of inducers include xylose, tetracycline, lactose and derivatives thereof including IPTG, arabinose and gluconate or a change of temperature. For example, if the promoter is controlled by a temperature-sensitive repressor, the promoter can be induced by increased temperatures.

The regulatory sequence or promoters associated with the feedback mechanism may comprise an endogenous bacterial promoter, or a polynucleotide homologous to such an endogenous promoter which retains the activity of the promoter i.e. is affected by the feedback mechanism. A non-naturally occurring promoter may be provided comprising responsive elements which respond to the feedback mechanism coupled to other regulatory sequences sufficient to operably express the reporter gene via the feedback mechanism.

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. Thus, a regulatory sequence such as a promoter, "operably linked" to a coding sequence is positioned in such a way that expression of the coding sequence is achieved under conditions compatible with the regulatory sequence.

The polynucleotide construct(s) may be provided as vector(s) for transformation of the bacterial host cell. The polynucleotide constructs may be provided on the same or different vectors. Vectors may be used to replicate the vectors in a compatible host cell. The vectors may be for example plasmid vectors provided with an origin of replication and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes such as ampicillin or chloramphenicol resistance genes for selection in bacterial cells.

In an alternative aspect, the invention also relates to a host cell transformed, conjugated or transduced with first and second polynucleotide constructs for the

expression of the first and second reporter genes.

The assay of the invention is used to screen for compounds which modulate synthesis or activity of the bacterial essential protein. Any suitable format may be used for the assay for identifying a modulator of bacterial protein activity (the target protein). The way in which the assay is carried out will depend in part of the nature of the first and where present second reporter genes. In some instances it may be necessary to divide a sample containing the host cells following administration of the test substance in order to monitor separately for first and second reporter gene activity. The host cells are preferably bacterial cells and may be selected from *Bacillus subtilis, E coli, Salmonella, Streptococcus, Staphylococcus etc.* The conditions of the assay are selected such that the host bacterial cell may grow in the absence of the test substance. Preferably the assay is carried out under conditions which allow for the feedback mechanism to operate if there is an alteration of the synthesis or activity of the target protein such that the reporter gene is expressed.

Additional control experiments may be appropriate. The progress of the assay can be followed in the presence and in the absence of the test substance. Known target protein modulators, may be used as positive controls in order to show a comparable or similar effect in a test substance. Known antibiotics may be tested to demonstrate their effect on the target protein. Additional assays may be carried out for example on mammalian cells or in a mammalian host to check that the test substance does not show adverse side effect on such host cells.

Suitable test substances which can be tested in the above assays include combinatorial libraries, defined chemical entities, peptide and peptide mimetics, oligonucleotides and natural product libraries. such as display (e.g. phage display libraries) and antibody products.

Test substances may be used in an initial screen of, for example, ten substances per reaction, and the substance of these batches which show inhibition or activation tested individually. Test substances may be used at concentrations from 1μM to 10mM, preferably from 1μM to 100μM, more preferably from 1μM to 10μM. Complex mixtures of natural origin (e.g. filtrates from bacterial cultures, or plant extracts) may be used.

Inhibitors of target bacterial protein activity may be used to restrict the growth of bacteria. Such inhibitors may be used to treat bacterial conditions in humans or animals and thus may be used as antibiotics to treat such bacterial infection.

Such inhibitors may be administered in a variety of dosage forms. Thus, they can be administered orally, for example as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules. The inhibitors may also be administered parenterally, either subcutaneously, intravenously, intramuscularly, intrasternally, transdermally or by infusion techniques. The modulators may also be administered as suppositories. A physician will be able to determine the required route of administration for each particular patient.

The formulation of a modulator for use in prophylaxis or treatment will depend upon factors such as the nature of the exact modulator, whether a pharmaceutical or veterinary use is intended, etc. A modulator may be formulated for simultaneous, separate or sequential use.

A modulator of target protein activity is typically formulated for administration in the present invention with a pharmaceutically acceptable carrier or diluent. The pharmaceutical carrier or diluent may be, for example, an isotonic solution. For example, solid oral forms may contain, together with the active compound, diluents, e.g. lactose, dextrose, saccharose, cellulose, corn starch or potato starch: lubricants, e.g. silica, talc, stearic acid, magnesium or calcium stearate, and/or polyethylene glycols; binding agents; e.g. starches, arabic gums, gelatin, methylcellulose, carboxymethylcellulose or polyvinyl pyrrolidone; disaggregating agents, e.g. starch, alginic acid, alginates or sodium starch glycolate; effervescing mixtures; dyestuffs; sweeteners; wetting agents, such as lecithin, polysorbates, laurylsulphates; and, in general, non-toxic and pharmacologically inactive substances used in pharmaceutical formulations. Such pharmaceutical preparations may be manufactured in known manner, for example, by means of mixing, granulating, tabletting, sugar-coating, or film coating processes.

Liquid dispersions for oral administration may be syrups, emulsions and suspensions. The syrups may contain as carriers, for example, saccharose or

saccharose with glycerine and/or mannitol and/or sorbitol.

Suspensions and emulsions may contain as carrier, for example a natural gum, agar, sodium alginate, pectin, methylcellulose, carboxymethylcellulose, or polyvinyl alcohol. The suspensions or solutions for intramuscular injections may contain, together with the active compound, a pharmaceutically acceptable carrier, e.g. sterile water, olive oil, ethyl oleate, glycols, e.g. propylene glycol, and if desired, a suitable amount of lidocaine hydrochloride.

Solutions for intravenous or infusions may contain as carrier, for example, sterile water or preferably they may be in the form of sterile, aqueous, isotonic saline solutions.

A therapeutically effective amount of a modulator is administered to a patient. The dose of modulator may be determined according to various parameters, especially according to the substance used; the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. Again, a physician will be able to determine the required route of administration and dosage for any particular patient. A typical daily dose is from about 0.1 to 50 mg per kg, preferably from about 0.1mg/kg to 10mg/kg of body weight, according to the activity of the specific inhibitor, the age, weight and conditions of the subject to be treated, the type and severity of the infection and the frequency and route of administration. Preferably, daily dosage levels are from 5 mg to 2 g.

A high throughput screen runs in the following way. A strain of *B. subtilis* is constructed containing two reporter genes encoding enzymes or proteins that can be detected, such as β-galactosidase and β-glucuronidase. Reporter genes capable of being expressed in *Bacillus* species are well known and documented in the literature. Reporter genes are preferably chosen so that their products can be readily assayed simultaneously. Green fluorescent protein has the advantage that its intrinsic fluorescence allows the protein to be assayed by direct fluorimetric measurement. *LacZ* has been used for more than 10 years with great success in *B. subtilis* and there is a range of useful substrates that generate coloured or fluorescent products upon hydrolysis by β-galactosidase. The *uidA* gene of *E.coli* has recently been harnessed for similar purposes, and the range of substrates available for the gene product, β-

glucuronidase is similar to that for β-galactosidase. Two different fluorogenic substrates may be used to assay the activities of the two reporters simultaneously in a single reaction. One reporter is fused to a sequence that causes increased expression in the absence of the desired function (e.g. gyrase); the other to a sequence that gives unchanged or decreased expression. The dual reporter strain is grown in an appropriate medium, dispensed into a vessel allowing large scale screening, such as the wells of microtitre plates. Each well would contain at least one test compound. The cells would be incubated for an appropriate time period (preferably two or more cell doublings), after which (if necessary, depending on the reporters used) a reaction cocktail allowing simultaneous assay of the two reporter enzymes would be added. The assays might be based on enzyme substrates giving e.g. chemiluminescent or fluorescent or coloured products. For fluorogenic substrates the presence of either or both enzymes may be detected simultaneously by a fluorimeter set to receive two different appropriate wavelengths. In the example described above, a positive response, in terms of inhibition of DNA gyrase, would be indicated by increased activity of β-galactosidase and decreased β-glucuronidase. Compounds eliciting such a response would be potential antimicrobial agents with DNA gyrase as the likely target.

Alternatively, the chemicals could be individually spotted onto a lawn of cell plated on a growth medium containing colorigenic or fluorogenic substrates. Chemicals eliciting an appropriate response would be detectable by their enhancement or inhibition of enzyme activity in the vicinity of the spot.

In principle, there are many essential genes in bacteria that are likely to be sufficiently different from mammalian cells to provide the selective toxicity needed for a useful antibiotic. Moreover, it is well known that mutations affecting central metabolic functions, such as nucleotide precursor synthesis, lead to attenuation of virulence, because they perturb the ability of the pathogen to grow in host cells or tissues. So this approach may provide not only antibiotics but also drugs that attenuate virulence and which will act synergistically with antibiotics. The strategy is especially important because it could be applied to any gene of interest, even though the precise function of the gene is not known.

The present invention also relates to the use of regulatory sequences or promoters which are not necessarily the promoter of the target gene itself but a promoter which is up-regulated in response to a decrease in the synthesis or activity of the target gene. The invention also provides a method for identifying such a regulatory sequence by looking for differential expression of a gene under the control of a regulatory sequence when the synthesis or activity of an essential protein is altered. In addition, an assay of the invention may involve monitoring differential expression of the gene affected by the feedback mechanism.

Identification of differential expression of a gene under control of the regulatory sequence may be carried out in a number of different ways. In a first embodiment, a library is prepared comprising fragments of the chromosome of a bacterial gene, each fused to a reporter gene. Such constructs are used to transform bacteria. The synthesis or activity of the essential target gene is then altered and expression of the reporter gene is monitored. Those constructs which show upregulation of the reporter gene are then further analysed to identify those regulatory sequences which are upregulated in response to an alteration of the synthesis or activity of the essential target gene. In an alternative embodiment, differential expression maybe monitored using a gene array of nucleotide sequences from the genes of the organism and analysing which genes are upregulated in response to alterations in the synthesis or activity of the target protein. Alternately, such differential expression could be analysed by recovering proteins from the organism and separating the recovered proteins to observe changes resulting from alterations in the synthesis or activity of the target gene. Promoter or regulatory sequences associated with such genes or proteins whose expression is altered in response to alterations in the synthesis or activity of the essential target gene may then be used in accordance with an assay of the invention.

In an assay of the invention, a gene whose expression if affected by alternations in the expression or activity of the target essential protein is selected. Bacterial cells are incubated in the presence and absence of a test substance and differential expression of the gene is investigated by one of the methods outlined above.

When the regulatory sequence is not a promoter of the target gene, then the invention also provides a method of identifying a regulatory sequence, which method comprises the steps of:-

i) selecting a target gene in a chromosome of an organism wherein expression of the target gene is subject to a feedback mechanism,

ii) altering the synthesis or activity of an expression product of the target gene, and

iii) observing a corresponding change in activity of a regulatory sequence associated with the said feedback mechanism,

wherein there is introduced into the chromosome of the organism a reporter gene under the control of the regulatory sequence, and step iii) is performed by observing a corresponding change in the expression of the reporter gene.

Step ii) of that method preferably involves controlling the expression of the target gene through the use of a genetic construct containing a known repressor or promoter sequence so as to modulate a level of an expression product via exposure of the cells to an external chemical or physical inducing factor; or isolating a conditional mutation in the gene, which allows activity of its product only under certain conditions, such as low or high temperature.

In one method there is introduced into the host organism a reporter gene under the control of the regulatory sequence, and step iii) is performed by observing a corresponding change in the expression of the reporter gene. Preferably there is provided a library of constructs of potential regulatory sequences associated with the said feedback mechanism, each potential regulatory sequence fused to a reporter gene. Preferably iii) is performed by genetically modifying aliquots of cells of the organism by introducing into the chromosome of the cells of each aliquot a different construct of the library. The genetic modification may simply comprise introduction of a plasmid containing a construct. The potential regulatory sequences are preferably DNA fragments of approximately 50-1000 bp of the genome of the organism. A library would preferably contain at least 10 different constructs. Preferably the DNA fragments of the library comprise the entire genome of the organism.

Another method involves providing an array of DNA sequences of genes of the organism and monitoring for differential mRNA expression in the presence of functional inhibition of the target gene. Preferably the array is of DNA sequences taken from each of the genes of the organism and provided in spots at spaced locations on a surface of a support. Cells of the organism are incubated under conditions which permit expression of the target gene, RNA is recovered and if desired converted to cDNA. Either the RNA or the cDNA is applied to the array under hybridisation conditions. and a hybridisation pattern is noted. Another aliquot of cells of the organism is incubated under conditions to alter or prevent expression of the target gene. RNA is recovered and RNA or cDNA is applied to the array under hybridisation conditions in order to generate a hybridisation pattern different from the previous one. The differences are noted and are indicative of genes and their regulatory sequences which are involved in a feedback mechanism associated with the target gene. Such gene expression arrays have been described in the literature and used to investigate feedback mechanisms of micro-organisms, but not, it is believed, for the purpose of identifying regulatory sequences for use in screening for antibiotics. (DeRisi, J L *et al*, 1997).

In yet another method. step iii) is performed by recovering proteins from cells of the organism, separating the recovered proteins and observing a corresponding change in concentration of at least one individual protein. Thus a first aliquot of cells of the organism is cultured under conditions to permit expression of the target gene. Proteins in the cell are recovered and separated e.g. by use of a two dimensional electrophoresis gel. This technique is well known in the literature (Anderson, N L *et al*. 1998) and gives rise to a pattern where the identity of each protein is known or can be determined. Then another aliquot of the cells is incubated under conditions to prevent expression of the target gene. Recovered proteins are separated by electrophoresis to produce a pattern different from the first. The differences are observed and are indicative of genes and regulatory sequences that are involved in a feedback mechanism associated with the target gene.

The first of these three methods involves a substantial capital investment to create a library of constructs of potential regulatory sequences each fused to a

reporter gene: but it should be effective to identify any or all regulatory sequences involved in the feedback mechanism associated with the target gene. The second method using gene expression arrays, avoids the expense of creation of a library, and should be effective to pick up regulatory sequences involved in feedback mechanisms where transcription is altered. The third method, using "proteomics" is relatively simple and cheap, but may not be effective to pick up regulatory sequences where the feed back mechanism involves mRNA synthesis or protein stability or activity.

Use of these methods permits regulatory sequences, involved in feedback regulation of a target gene but which are not promoters of the target gene, to be identified. Once this has been done, it is a simple matter to construct cells as defined, containing an exogenous reporter gene under the control of the regulatory sequence, and to use these cells in a whole cell assay for screening compounds for antibiotic or other biological activity.

The organism is preferably a bacterium e.g. *Bacillus subtilis*, although other prokaryotes and even simple eukaryotes such as yeasts are envisaged. It may be convenient to use an organism whose genome is sufficiently small that it can be chopped up into random fragments of convenient size which constitute a library of potential regulatory sequences, with the number of potential regulatory sequences of the library not being impractically large. This is a property possessed by bacteria and possibly also by yeasts.

For any given target gene, there is thus a good chance that somewhere in the genome there will be a regulatory sequence the activity of which is enhanced or reduced by lack of the target function. In *B. subtilis* it is straightforward to turn off any target gene by making genetic constructions in which the gene can be repressed (e.g. by use of the IPTG-inducible P_{spac} promoter (Yansura & Henner, 1984), or the xylose-inducible promoter, P_{xyl} (Feucht *et al*, 1996)). When the inducer compound is taken away, expression of the gene is blocked and the target function is depleted from the cell. As discussed above, this is likely to result in specific induction of one or more genes. To find the promoter or regulatory sequences for such a gene, a large (>10,000) random collection of short (approximately 50 to 1,000 bp) DNA sequences

from the chromosome of *B. subtilis* or a related organism are fused to an appropriate reporter gene (e.g. *lacZ*). Methods known in the art are available to achieve this (reviewed by Errington, 1990). Each member of the collection of fusions is then introduced into a cell of a strain of *B. subtilis* that had been genetically engineered to allow depletion of the target function. A regulatory sequence exhibiting the desirable property of modulating reporter gene expression upon depletion of the target function would provide a means of screening for compounds which are specific modulators of the target gene function. For example, if the gene encoding one of the subunits of DNA gyrase (*gyrA*) is depleted, the resultant change in DNA supercoiling in the cell should result in increased expression from the promoter of the *gyrA* gene. Some random fragments of DNA (e.g. one containing the *gyrA* promoter itself), when fused to a reporter gene and introduced into the cells in which gyrase has been depleted, would give rise to increased reporter activity. Isolating the gyrA promoter fragment (or some similarly behaving regulatory sequence), and fusing it to a reporter gene, gives a genetic construct that would respond, with increased expression, to inhibition of DNA gyrase by chemical inhibitors (i.e. potential antibiotics) (Menzel & Gellert, 1983). In any operational screen, it is preferable to include a second reporter gene, encoding a non-gyrase-dependent promoter fragment, to control for non-specific inhibitors of gene expression. Alternatively growth of the culture could be followed by, for example measurement of optical density and correction made for non-specific effects on culture growth. In principle, it might sometimes be possible to find, in the random collection, a DNA sequence which, when fused to the reporter and depleted for e.g. the *gyrA* product, would respond with decreased expression. Such a reporter would provide a control reporter that would most likely increase the specifity of the assay for inhibitors of DNA gyrase.

The general strategy may be applicable to other organisms. Yeast genetics should be facile enough to allow it to be used. This opens up the possibility of identifying compounds that inhibit specific eukaryotic functions; at least those that are conserved in yeast. The yeast strain may first have its endogenous gene replaced with the equivalent human gene to make the screen more direct and specific.

Example 1

A plasmid (pAT1) containing the N-terminal-coding region of the *gyrA* gene of *B. subtilis* is transformed into *B. subtilis* so as to create strain 2842. In this strain, the plasmid is integrated into the chromosome by homologous recombination, resulting in partial duplication of the *gyrA* gene. The functional copy of *gyrA* that remains has been placed under the control of the repressible P_{spac} promoter, so that removal of IPTG from a growing culture results in depletion of GyrA protein from the cells. To improve the repression of the P_{spac} promoter, a second, autonomously replicating plasmid also was introduced, p65, providing multiple copies of the *lacI* repressor gene (giving strain 2844). GyrA is one subunit of the essential DNA gyrase protein, so that in the absence of IPTG, the culture growth was strongly impaired Fig 1A. Integration of plasmid pAT1 also places a *lacZ* reporter gene under the control of the natural promoter for *gyrA*. The level of expression of this reporter was increased in the absence of IPTG, compared with the control culture in which IPTG remains present (Fig.1B) indicating that the promoter is subject to negative feedback regulation. Reduction in the availability of GyrA protein results in an increase in transcription from the *gyrA* promoter.

In principle, strains 2842 and 2844 provide a means of detecting inhibitors of DNA gyrase, since treatment of the cells with such compounds should similarly elicit increased relative expression of the *lacZ* reporter gene. To demonstrate this, strain 2844 was grown in the presence of IPTG and treated with a range of concentrations of a known inhibitor of DNA gyrase, nalidixic acid. As shown in Fig. 2, at a range of concentrations of nalidixic acid, from 2 μg/ml through 16 μg/ml, the specific activity of β-galactosidase (encoded by the *gyrA-lacZ* reporter gene) was increased to about 3-fold greater than that of the untreated cells.

To show that the assay strain could be used to screen compounds for activity against DNA gyrase in high throughput format, samples from a culture of strain 2844 were introduced into the wells of a 96-well microtire plate to which a series of antibiotics with a range of known modes of action had been added. Each antibiotic was added in the form of a 2-fold dilution series. After allowing 2 h for growth, the β-galactosidase specific activity was measured, using culture optical density (OD_{600})

to correct for reductions in cell growth due to the antibiotic action. Figures 3A, B and C plot the activity against the well number. The concentration of antibiotic in well 1 is as follows in μg/ml Fig 3A: polymyxin: 256, proflavin 165, trimethoprim 128, vancomycin 8, nalidixic acid 128; Fig 3B: ampicillin 128, carbenicillin 1024, chloramphenicol 128, nalidixic acid 128, rifampicin 64; Fig 3C: bacitracin 1024, chlorhexadine 128, monensin 128, novobiocin 128, phosphomycin 750. Wells 2-12 then follow the 2-fold dilution series. As shown in Fig. 3A, B & C, both nalidixic acid and novobiocin stimulated expression from the gyrA promoter in the assay plates, whereas chloramphenicol, kanamycin, rifampicin, carbenicillin, polymyxin B, and vancomycin did not show significantly higher specific activities than the untreated control cells. Interestingly, proflavine and trimethoprim, which affect other aspects of DNA metabolism, also registered as positives in the assay. We conclude that the feedback regulation exploited with the assay strain should be useful in identifying inhibitors of DNA gyrase and possibly compounds acting on other facets of the DNA replication machinery.

ADDITIONAL REFERENCES

Menzel, R. & Gellert, M. (1983) Regulation of the genes for *E.coli* DNA gyrase: homeostatic control of DNA supercoiling. Cell 34, 105-113.

Yansura, D.G. Henner, D.J. (1984) Use of the *Escherichia coli* lac repressor and operator to control gene expression in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA 81, 439-443.

Errington, J. (1990) Gene cloning techniques. In: Molecular biological methods for Bacillus. (Eds C.R. Harwood & S.M. Cutting), pp. 175-220. Chichester, UK.: Wiley.

Henkin, T.M. (1994) tRNA-directed transcription antitermination. Mol. Microbiol. 13, 381-387.

Feucht, A., Magnin, T., Yudkin, M.D. &Errington, J. (1996) Bifunctional protein required for asymmetric cell division and cell-specific transcription of *Bacillus subtilis*. Genes Devel. 10, 794-803.

Lu, Y., Turner, R.J. & Switzer, R.L. (1986) Function of RNA secondary

structures in transcriptional attenuation of the *Bacillus subtilis* pyr operon. Proc. Natl. Acad. Sci. USA 93. 14462-14467.

DeRisi *et al*. 1997. Exploring the metabolic and genetic control of gene expression on a genomic scale. Science. 278, 680-686.

Anderson, N L. *et al*, Proteome and Proteomics: New Technologies, new concepts and new words. Electrophoresis, 1998, August; 19 (11): 1853-61.

CLAIMS

1. A method for identifying a regulatory sequence which is affected by a feedback mechanism on alteration of synthesis or activity of a bacterial essential protein comprising:

i) providing a bacterial cell having a reporter gene under the control of a candidate regulatory sequence;

ii) selecting a target essential protein which is expressed in the organism;

iii) altering the synthesis or activity of the essential protein;

iv) monitoring expression of the reporter gene; and

v) determining thereby whether the candidate regulatory sequence is affected by a feedback mechanism responsive to alteration of the synthesis or activity of the essential protein.

2. A method of identifying a regulatory sequence whose activity is affected by a feedback mechanism or an alteration of the synthesis or activity of an essential bacterial protein comprising:

(a) monitoring expression of a bacterial gene in a bacterial host cell in the presence of normal and altered synthesis or activity of the essential protein;

(b) identifying differential gene expression in the presence of normal and altered synthesis or activity of the essential protein; and

(c) identifying thereby a regulatory sequence whose activity is affected by the feedback mechanism.

3. A method according to claim 2 wherein step (b) comprises providing an array of nucleotide sequences of the genes of the bacterial cell, recovering polynucleotide material from the cells of the organism and applying such polynucleotide material to the array and monitoring for hybridisation of the bacterial nucleic acid material to the array.

4. A method according to claim 2 wherein step (b) comprises recovering and separating proteins from the bacterial cell and monitoring for a change in concentration of a protein in the presence of normal and altered synthesis

or activity of the essential protein.

5. A method for identifying a modulator of a bacterial essential protein comprising:

i) providing a bacterial host cell which expresses the essential protein and having a polynucleotide construct comprising a regulatory sequence operably linked to a reporter gene wherein the regulatory sequence is associated with a feed back mechanism responsive to alteration in the synthesis or activity of the essential protein and is identified according to the method of any one of claims 1 to 4;

ii) contacting a test substance with the host cell; and

iii) monitoring expression of the reporter gene to determine thereby whether the said substance modulates the synthesis or activity of the essential protein.

6. A method according to claim 5 wherein the essential protein is involved in cell wall synthesis, teichoic acid synthesis, DNA replication, RNA synthesis, cell division, chromosome segregation, translation or lipid synthesis.

7. A method according to claim 5 or claim 6 wherein inhibition of the essential protein up-regulates expression of the reporter gene from the regulatory sequence.

8. A method according to any one of claims 5, 6 or 7 wherein the regulatory sequence has the activity of a promoter for the gene encoding the essential protein and inhibition of the essential protein up-regulates expression from its promoter.

9. A method according to any one of claims 5, 6 or 7 wherein the regulatory sequence has the activity of a promoter for a gene which does not encode the essential protein but which is up-regulated via the feedback mechanism in response to alterations to the synthesis or activity of the essential protein.

10. A method according to any one of claims 5 to 9 wherein the reporter gene comprises a gene which is up-regulated in response to alterations in the synthesis or activity of the essential protein and step (iii) comprises monitoring for differential expression of the gene in the presence or absence of the test substance.

11. A method according to any one of claims 5 to 10 wherein the bacterial cell is provided with a second polynucleotide construct comprising a promoter operably linked to a second reporter gene and the method further comprises monitoring expression of the second reporter gene.

12. A method according to any one of claims 5 to 11 comprising determining whether the test substance demonstrates specific inhibition of the essential protein.

13. An inhibitor of a bacterial essential protein identifiable by the method of any one of claims 5 to 12.

14. An inhibitor of a bacterial essential protein identified according to the method of any one of claims 5 to 12.

15. An inhibitor according to claim 13 or claim 14 for use in a method of treatment of a human or animal body.

16. An inhibitor according to claim 15 for use as an antibiotic.

17. A pharmaceutical composition comprising the inhibitor of any one of claims 13, 14 or 15 and a pharmaceutically acceptable carrier.

18. A method of inhibiting bacterial growth comprising identifying an inhibitor of a bacterial essential protein according to any one of claims 5 to 12, and contacting a compound so identified with bacteria to be inhibited.

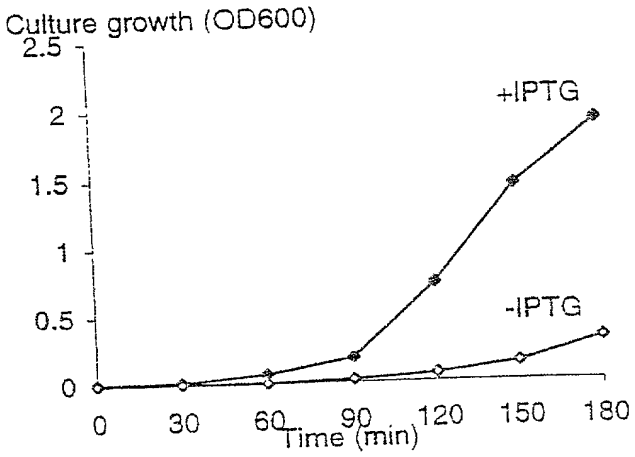


Fig. 1A

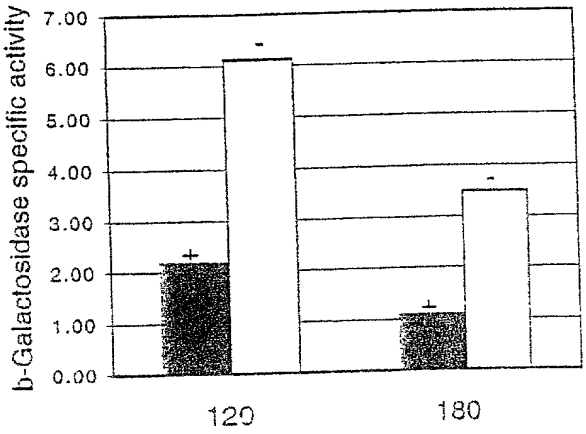


Fig. 1B

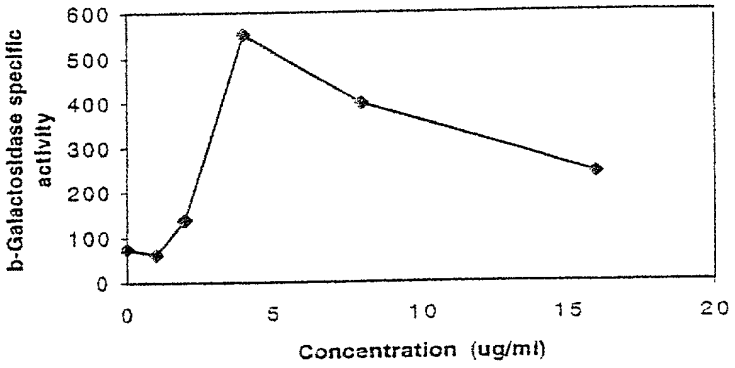
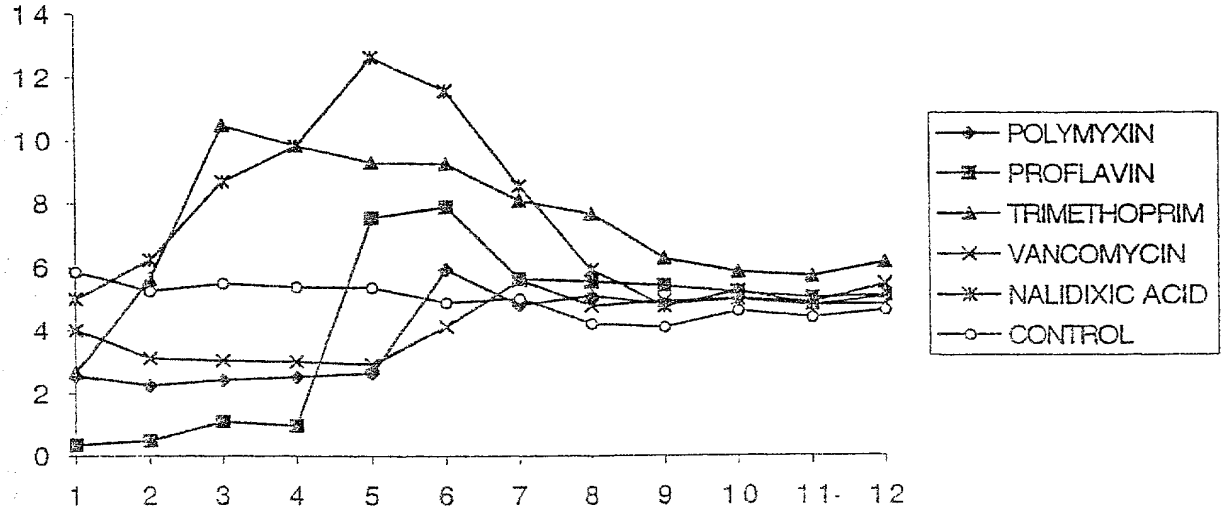


Fig. 2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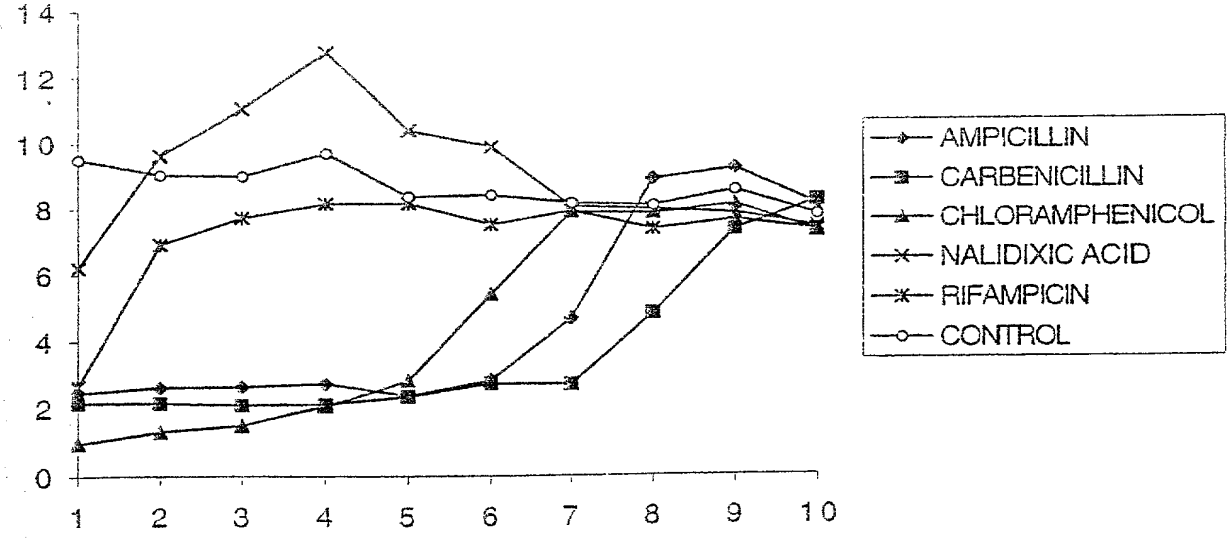
NALIDIXIC ACID
CONTROL

Fig. 3A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NALIDIXIC ACID
RIFAMPICIN
CONTROL

Fig. 3B

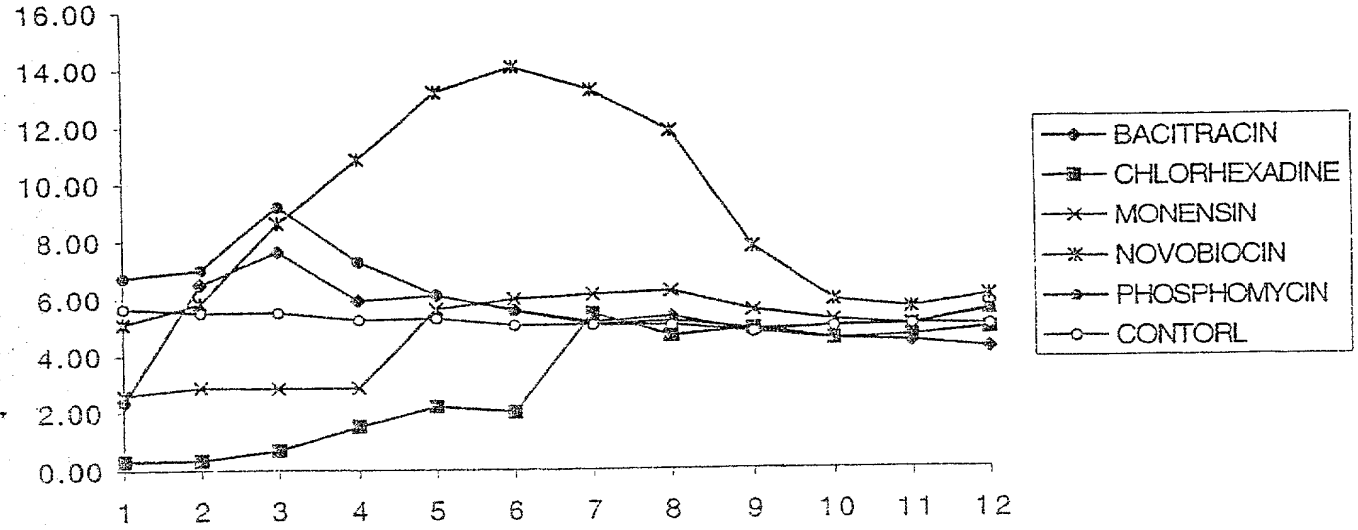

Fig. 3C

Application Data Sheet

Application Information

Application number::	09/869,179
Application Type::	Regular
Subject Matter::	Utility
Suggested Group Art Unit::	N/A
CD-ROM or CD-R?::	None
Sequence submission?::	NO
Computer Readable Form (CRF)?::	No
Title::	METHOD OF SEQUENCE IDENTIFICATION
Attorney Docket Number::	HO-P02222US0
Request for Early Publication?::	No
Request for Non-Publication?::	No
Small Entity?::	Yes
Petition included?::	No
Secrecy Order in Parent Appl.?::	No

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Continuity information:	International application no. PCT/GB99/04352

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DECLARATION (CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

As a below named inventor(s), I/we declare that:

The declaration is directed to:

☐ The attached application, or
International Application No. PCT/GB99/04352, filed 12/22/99
☒ Application No. 09/869,179, filed on June 21, 2001.
☐ as amended on _____ (if applicable).

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including material information which became available between the filing date of the prior application and the National or PCT international filing date of the continuation-in-part application, if applicable; and

All statements made herein of my/our own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME(S) OF INVENTORS:

Inventor one: Jeffrey Errington

Signature: _____ Citizen of: United Kingdom

Inventor two: _____

Signature: _____ Citizen of: _____

Inventor three: _____

Signature: _____ Citizen of: _____

Inventor four: _____

Signature: _____ Citizen of: _____

☐ Additional inventors are being named on _____ additional form(s) attached hereto.

Declaration for Utility or Design Patent Application

I hereby certify that this correspondence is being deposited with the U.S. Postal Service as Express Mail, Airbill No. EL D984944577US, in an envelope addressed to: Commissioner for Patents, Washington, DC 20231, on the date shown below.

Dated: Mar. 11, 2002 Signature: Susan Hunter (Susan Hunter)

NB:- SEE THE APPLICATION DATA SHEET FOR FURTHER INFORMATION